Novel Antiviral Compound Shows In-Vivo Efficacy Against Cytomegalovirus

A Senior Honors Thesis

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by

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Abstract

Widespread use of Ganciclovir (GCV) therapy has led to resistant strains of cytomegalovirus (CMV), prompting development of new antiviral compounds. A novel duplex compound of Zidovudine and Foscarnet (N3) has shown excellent *in-vitro* activity against both GCV sensitive and resistant HCMV strains. To begin *in-vivo* efficacy studies, we chose to use a well-characterized murine model of CMV (MCMV) infection. We confirmed *in-vitro* activity of N3 against MCMV using infected fibroblasts. QRT-PCR demonstrated that N3 controlled *in-vitro* DNA replication at 48 or 72hrs pi. To test *in-vivo* efficacy, cohorts of BALB/c mice infected with 5 X 10^4 PFU Smith strain MCMV received daily intraperitoneal dosing of N3 (5-50mg/kg), Ganciclovir (GCV, 10mg/kg), Foscarnet (PFA, 45-90mg/kg), or saline. N3-50mg and GCV showed comparable reduction of salivary gland infectious virus titers 14 days post infection, while N3-5mg did not control virus titers. Quantitative PCR (QRT-PCR) showed that GCV treatment reduced viral DNA load in salivary glands, but N3 did not. Similarly, lungs showed no significant reduction in viral DNA load for N3-50mg. In contrast, N3 (all doses), PFA, and GCV similarly reduced hepatic viral DNA load compared to saline treated mice. Taken together, these data suggest some *in-vivo* antiviral effect of the novel N3 compound. Interestingly, in some tissues, N3 may inhibit viral growth without inhibiting DNA replication. Overall poor effect of N3 in organs outside the peritoneal cavity suggests a problem with drug bioavailability when administered via the intraperitoneal route. Further evaluation of N3 efficacy *in-vivo* and mechanism of action will be required.

Introduction

Cytomegalovirus (CMV) is a ubiquitous member of the beta-herpes virus family, infecting 60% of the US population by age 6, and nearly 90% during their lifetime (1). Although acute infection is common and usually controlled by the host, it does pose several threats. CMV is the most common viral cause of congenital birth defects in the United States (1). 40,000 children each year are born with congenital CMV infection that can lead to hearing loss and mental retardation. CMV is also of serious concern to immunosuppressed individuals (transplant, therapeutic, HIV, etc), causing CMV pneumonia, retinitis, gastroenteritis, encephalitis, disease similar to mononucleosis, and increased susceptibility to bacterial and fungal infection (2-4). Prior to development of antiviral therapy, CMV was a leading cause of morbidity and mortality in these patient populations.

Following primary infection, the virus enters a latent state that centers in myeloid progenitor cells of the bone marrow and, to a lesser extent, other tissues (5, 6). During latency, viral DNA incorporates itself into host cells by forming and maintaining episomes. CMV may exit latency by reactivation during times of immunosuppression. Once reactivated, disease can occur once again.

Unfortunately, there are still numerous hurdles for therapy of CMV. Unlike many other viruses, there are presently no effective vaccines that provide sterilizing immunity. Passive transfer using immunoglobulin from previously infected individuals has similarly failed to prevent infection (7). Because current pharmacologic therapies target active virus, these therapies become ineffective once CMV infections become latent. Thus, treatment is administered for severe primary infection, or for cases of viral reactivation.

Although currently available antiviral treatments are often life-saving, all have drawbacks. Ganciclovir (GCV) is the drug of choice for most CMV infections (8). GCV is a guanosine analog lacking a 3'OH that competitively incorporates into replicating viral DNA causing elongation disruption. Prior to incorporation, GCV is activated by the kinase produced by human CMV (HCMV) gene, UL97 (a conserved kinase, M97 is present in mice). Therapy with GCV does provide effective suppression of virus replication, but, in addition to the compound's toxicity, widespread use has led to resistant strains (9). Alteration of the UL97 kinase is often the cause of resistance (10).

Foscarnet (phosphonoformic acid, PFA), an organic analog of inorganic pyrophosphate, is an alternative therapy to GCV (8). PFA is another viral polymerase inhibitor that functions by blocking pyrophosphate loading of virally encoded DNA polymerase (product of gene UL54 in humans, M54 in mice). It is not activated by UL97 like GCV and therefore has activity against many GCV resistant strains. Unfortunately, PFA is nephrotoxic at the effective dose of 90mg/kg, which limits PFA's clinical utility.

These shortcomings demand new antiviral development capable of treating resistant strains without nephrotoxicity. It has been suggested that AZT, an antiviral thymidine analog, can be effectively used in some cases of CMV retinitis (11). Nonetheless, it is most often used in cases of HIV infection and there has been little study into its efficacy against CMV. Prof Schott (University of Tübingen, Germany) has

developed a novel duplex antiviral agent, designated N3 (fig. 1), which is a combination of PFA and AZT by glyceryllipid residue. This duplex is intended to provide synergistic effect of AZT and PFA, reducing the amount of PFA needed by half and reducing the possibility of nephrotoxicity. This novel compound has shown excellent *in-vitro* efficacy against CMV replication.

It is our hypothesis that N3 has antiviral action against CMV *in-vivo*. We utilized a murine model of CMV (MCMV) infection to investigate this hypothesis (12). With development of any drug, toxicity must be explored first in non-human subjects if possible. Because of ethical limitations, HCMV studies are difficult, and are often carried out using murine models due to similarities between the two CMV strains (13). A primary infection model was chosen to keep experiments as simple as possible in the initial trial. We hope to determine whether N3 is effective against MCMV *in-vivo*, and compare its effectiveness against GCV or PFA. Proving *in-vivo* efficacy of N3 will be necessary before clinical trials can be performed to test effectiveness against HCMV. Thus, these studies are an important first step towards the potential development of N3 as an effective treatment for HCMV infections.

Methods

In-Vitro Efficacy of N3 for MCMV

Cell culture-based experiments were used to examine the ability of N3 to inhibit viral DNA replication and to ensure activity against MCMV. 25, 50, 100, or 200µM doses of N3 were applied in a single dose to MCMV-infected NIH 3T3 fibroblasts. Infected cells were harvested at 24, 48, or 72hrs post infection and DNA was purified for quantitative real-time PCR (QRT-PCR) analysis of viral DNA content. A second *in-vitro* experiment was conducted in similar fashion; in this case, two titers of virus, 5 X 10^1 plaque forming units (PFU) per well and 5 X10^3 PFU/well, were dosed, and cell culture media was supplemented with a 100µM concentration of N3 and replaced daily with fresh media containing 100µM N3 prior to harvesting cells and purifying DNA.

PCR and Quantitative PCR

DNA was purified by QIAamp Tissue kits (QIAGEN GmbH, Hilden, Germany), eluted in 150µl of AE buffer (contained in the kit), and stored at -20°C until analysis. DNA was quantitated by measuring absorption at 260nm/280nm ($A_{260/280}$) using a Beckman DU 640B spectrophotometer. 500ng of DNA was amplified in a total volume of 25µl with 200nM of each DNA primer, 200nM of gene-specific fluorescent probe, and 12.5 of 2X Takara Premix Ex Taq (Roche Molecular Systems, Basel Switzerland).

Primer sets and fluorescence resonance energy transfer type probes for MCMV glycoprotein B (gB) and beta actin (ß-actin) quantitation were designed for the Cepheid SmartCycler (San Diego California). GB is a viral envelope protein that is always synthesized during MCMV replication. ß-actin DNA was used as a cellular transcript control, since it is expressed at constant levels under many conditions, including virus replication. During the single strand phase of PCR, the probe binds the complementary target sequence, and fluoresces. This emission is measured by the optical receptors of the machine and recorded. Once the emission intensity crosses a threshold preset to exclude random binding, the machine notes the number of cycles completed up to that point. Initial DNA quantity is inversely related to cycle count.

QRT-PCR was performed under the following program: initial denaturation 10 sec at 95°C, 50 cycles-denaturation 15s at 95°C, annealing 15s at 58°C with the optics on, and elongation 30s at 68°C. The short initial denaturation step was chosen per manufacturer instruction to optimize fully the hot-start Taq included in the Takara mix. Plasmids homologous to the sequence amplified by our primers were cloned for both the gB and ß-actin genes, and using serial dilution of known plasmid concentrations, standard curves were constructed for quantification of products obtained by QRT-PCR. Regression equations had high R^2 values (0.995 for gB and 0.997 for ß-actin), and were thus used for all subsequent copy number calculations. Real time PCR results are expressed as copies of gB/10E4 copies of ß-actin for liver and lung results. Salivary gland results are expressed in copies of gB/ß-actin

Sequences for real time primers/probes designed are as follows:

Gene/Direction	Forward	reverse	probe
ß-actin	attgtgatggactccggtga	agctcatagctcttctccag	caccacactgtcccatctac
CMV gB	tgtactcgaagggagagct	cgttcaccaccgaagacac	cgcctcgaacgtgttcagcctg

Infection Preparation

Female BALB/c mice (Harlan, Indianapolis, IN) 6-8 weeks of age were used in this study. Purified Smith strain MCMV was obtained from ATCC (Rockville, MD). Primary infection was achieved by intraperitoneal injection of mice with 5 X 10^4 PFU Smith strain MCMV. Groups (n=5) were randomly assigned to receive saline, GCV 10mg/kg/day, PFA 45 mg/kg/day, PFA 90 mg/kg/day, N3 5mg/kg/day, N3 10mg/kg/day, N3 25 mg/kg/day, or N3 50mg/kg/day. All doses were administered in 0.2cc of saline. A dose of 10mg/kg/day of GCV was chosen because this has been shown to be efficacious in preventing virus replication in mice (14). Because 90mg/kg/day is the recommended dose of PFA (15); and because previous research has indicated the lethal dose of N3 in mice to be 100mg/kg (Schott, personal communication); a dose of 50mg/kg/day of N3 was chosen to test whether a half dose of N3 is as effective as the standard dose of PFA. In addition, a series of lower N3 concentrations were also tested (see above).

Antiviral treatment was initiated the day after infection and mice were weighed several times during the course of the experiment. Frequent weighing was used as a measure of the drug's general effects on the mice. Mice were euthanized 14 days post-infection by cervical dislocation under inhalation anesthesia. To examine for antiviral effects on normal mouse kidney function, blood was collected to test for creatinine levels using an i-STAT (Heska Corporation, Friboug, Switzerland). Creatinine, a by-product of creatine phosphate breakdown in the muscles, is filtered by the kidneys. A high creatinine level in the blood would be an indication of renal toxicity. Serum was extracted from the blood by centrifugation, flash frozen in liquid nitrogen, and then stored at -80°C. Heart, lungs, salivary gland, spleen, liver, and kidneys were dissected aseptically and frozen in the same manner. Small sections were removed from each organ at the time of dissection, preserved in 10% formalin, and embedded in paraffin blocks for histological analysis.

In-Vitro Plaque Assay for Viral Quantitation

To assess the presence of infectious virus, plaque assays were performed on salivary gland and lung homogenates. Organ homogenates were made by removing a small sections, weighing them, and adding phosphate buffered saline (PBS) to create a 5% (weight:volume) solution. The organ and PBS solutions were then homogenized with an electric tissue homogenizer at 30,000 rpm. Additionally, salivary gland homogenates were sonicated 4 times for 3 seconds to ensure cell lysis. These lysates were clarified by centrifugation at 2000 rpm for 10 min, serially diluted in Dulbecco/Vogt Modified Eagle's Minimal Essential Medium (DMEM) plus 2% Newborn Calf Serum (NCS), and the supernatants placed on confluent monolayers of NIH 3T3 fibroblasts (ATCC) in six-well plates (35 mm wells). As a control for virus infection, an uninfected mock well was left on each plate. Plates were subsequently centrifuged at 1220 rpm for 30min and incubated for 2 hours at 37°C. After the incubation period, media was removed and replaced with DMEM containing 10% NCS and 1% low-melting agarose. Once the agar had solidified, plates were incubated at 37°C/5% CO_2 in cell culture incubators. 5 days

post-infection, plates were fixed in 10% formalin. Agar plugs were removed and plates were stained with crystal violet for enumeration. Dilutions containing approximately 10-100 plaques per well were used to determine virus titers. All virus titers enumerations were performed in duplicate.

Statistics

Student's T tests or Analysis of variance tests were performed using Graphpad software to ensure statistical significance.

Results

In-vitro Efficacy of N3 for MCMV

Initial *in-vitro* experiments showed N3 to be ideally effective at 100µM; in contrast, 200µM induced cellular cytotoxicity (cell lysis, data not shown). When media and drug were not replaced after the initial addition, replication was inhibited most effectively on the first day (data not shown). DNA levels steadily rose for subsequent days when infected cells were homogenized and viral DNA was quantified by QRT-PCR. In contrast, viral DNA replication was inhibited when drug and media were replenished daily (fig. 2). 100µM N3 was shown to completely inhibit MCMV at 5×10^1 PFU and significantly reduce viral DNA load at 5×10^3 PFU, in comparison to untreated infected cells.

General Response to N3 Compound

Mice tolerated most doses of N3 well. There were no noticeable side effects at 5, 10, or 25mg/kg/day of N3. At the highest dose of N3, 50mg/kg/day, subjects showed weight loss of approximately 8% total body weight (fig. 3) on average and appeared to have loose, shaggy coats. The livers of mice in this group also displayed blanched regions and their behavior was hyperactive (characterized by frequent abnormally high jumping and overreaction to injection). Creatinine testing showed no nephrotoxicity characteristic of PFA treatment. Creatinine levels were normal for all groups. Histology has not been completed at this time.

Liver Viral Load

QRT-PCR showed that viral DNA levels were reduced in the liver approximately eight-fold with N3 treatments of 5, 10, 25, or 50 mg/kg/day, in comparison to untreated mice. This reduction was comparable to GCV in the liver (fig. 4). An important note is that the lowest dose of N3, 5mg/kg/day, appeared to be as effective as GCV, PFA or any of the higher N3 concentrations. ANOVA suggested that treatment groups had significantly lower viral DNA titers than saline controls ($p=0.0001$), and individual students t-tests comparing each treatment group to saline controls showed that titers were significantly lower for all treatment groups ($p<0.05$). There were no significant differences between treatment group viral titers when compared individually.

Lung Viral Load

There was no significant difference between any treatment group and saline controls in lung viral DNA loads ($p>0.05$, fig. 5). In addition, plaque assays of lung homogenates did not produce any viable counts of infectious virus. This unexpected result suggests that low amounts of infectious virus made it into the lungs after infection,

Salivary Gland Viral load

Plaque assays showed N3 50 mg to have comparable efficacy in reducing infectious particle formation when compared to GCV; and both showed a ten-fold reduction in PFU compared with saline or N3 5mg ($p<0.05$, fig. 7). Surprisingly, viral DNA load was not reduced in the salivary gland by N3 treatment, in contrast to GCV, which significantly reduced viral DNA load compared to saline controls ($p<0.05$, fig 6). Viral DNA load data produced by QRT-PCR indicates the quantity of viral DNA present in the organ. Viral titers were somewhat higher than typical for MCMV, but the repeat data indicates log reductions that are in line with ten-fold dilution series prepared and run in duplicate. This would seem to indicate that the atypically high titers could be attributed to a repeatable and relative counting error by the researcher.

Discussion

This study demonstrates that N3 is capable of inhibiting MCMV *in-vitro*, and suggests that it may be effective *in-vivo*. Liver data indicate that N3 inhibits infectious virus particle titers and viral DNA load. On the other hand, bioavailability appeared to be a problem in the lung and salivary gland. Data for these organs did show some anti-viral effect, but, due to intraperitoneal dosing, it is not clear if N3 was active in these organs or just prevented spread of live virus to these organs from the original intraperitoneal infection location. Nevertheless, it is our conclusion that N3 shows promise in the treatment of CMV infection and is worthy of future study.

In-vitro experiments suggested N3 was effective against MCMV. We found that 100μM was the most effective *in-vitro* dose that did not cause cytotoxic effects to the fibroblasts. We also demonstrated that *in-vitro* effectiveness of N3 decreases without replenishment in culture media. When combined with data indicating reduction of viral load in the liver, we concluded that N3 has potential efficacy *in-vitro* and *in-vivo* against MCMV. Furthermore, these experiments indicate that the mouse model is a viable route for future testing of N3 as a potential antiviral drug used to treat CMV infections.

Our QRT-PCR results indicated that intraperitoneal dosing was effective in controlling virus replication in the liver. An 8-fold reduction of viral load suggested that N3 is effective in inhibiting CMV replication *in-vivo*, even at low doses. Unfortunately, N3 appears to show toxicity to the liver at the 50mg dose. The blanched regions of the liver combined with the abnormal behavior of the mice receiving this N3 dose is not conclusive, but does suggest that 50mg/kg/day of N3 delivered intraperitoneally can cause liver damage. Histologic evaluation may provide some insight into the effect on the liver, and other organs tested, even though other organs showed no gross abnormalities.

Although there was a trend toward efficacy of N3 inhibiting CMV replication in the lungs, with the most effective dose being 50mg/kg, these differences were not statistically significant. This pulmonary effect might be a consequence of intraperitoneal N3 preventing spread of virus from the infection site. There is no strong evidence yet that N3 reaches organs other than liver when administered intraperitoneally, but the effect of N3 in liver may be strong enough to preclude spread to these distant organs. Similar effect was seen for intraperitoneal GCV and PFA, which did not show a significant effect in the lungs. In some ways, this was poor experimental design, because it is well known that GCV and PFA are poorly available by any route other than intravenous. Nonetheless, intraperitoneal control drugs were appropriate comparators for intraperitoneal N3, and measuring serum concentrations of N3 after intraperitoneal administration will help to answer this question.

Salivary gland results also showed contradictory findings. Plaque assay data indicated that N3 is capable of reducing viral titers comparable to GCV. On the other hand, our QRT-PCR results imply that N3 is not inhibiting production of viral DNA. QRT-PCR does not discriminate between viable or nonviable virus, nor does it indicate if viable virus is capable of producing an infection. Because *in-vivo* mechanisms of action of N3 have not been elucidated, it is possible that N3 controls MCMV by an additional mechanism other than inhibition of DNA replication. It is also possible that like lungs, N3 is not reaching the salivary gland, due to first pass effect or poor bioavailability, and

that the viral control seen here was again a consequence of prevention of dispersion of the infection beyond the liver.

Bioavailability is a measure of the quantity of a compound that can reach end organs. When administered via an intraperitoneal route, N3 may not have adequate bioavailability because it may be metabolized during passage through the hepatic portal system of the liver. This "first-pass effect," could result in extensive reduction of serum and distant tissue drug concentrations. Previous studies have shown that blocking the free –COOH of N3 by an $–OC_2H_5$-ethyl ester group or blocking the pyrophosphate binding site of the viral polymerase would completely destroy antiviral activity of N3 *in-vitro* (Schott, personal communication). This could be one of many possible mechanisms of hepatic metabolism. Additional studies are necessary to discover the source of the reduction of N3 antiviral activity in distal organs. The first and simplest study will be to measure N3 serum concentrations after 2 weeks of therapy. In addition, experiments using alternate routes of administration are ongoing, and will hopefully demonstrate improved bioavailability and *in-vivo* effect.

Future research will expectantly show that N3 has several possible uses. In addition to *in-vitro* data suggesting efficacy against GCV resistant strains of CMV, N3 may be valuable to HIV/CMV dually infected individuals. In the dually infected patients, treatment of concomitant infections, such as herpes simplex virus, has been shown to reduce HIV-1 RNA levels (16). The AZT subsection of the N3 molecule may provide concurrent action against HIV increasing overall utility of N3. In theory, enzymatic cleavage should release several different antiviral metabolites e.g. AZT, Foscarnet, lipophilic AZT, and Foscarnet derivatives. Lipid prodrugs of phosphonoacids like 1-O-octadecyl-sn-gylcero-3-phosonoformate (to which N3 can be compared) have been shown to enhance antiviral activity of PFA in HIV, HSV, and HCMV-infected cells *in-vitro*. This is because of the increased uptake by host cells, where intracellular enzymes then convert prodrug to an active form (17). Lipophilic AZT provides an added benefit in increased cellular membrane passage much like the lipid prodrug of PFA. These products are beneficial to the action of the drug and could improve the overall efficacy of N3, despite the fact that N3 pharmacokinetics dominate the effects of any single compound. This is advantageous since the drug is a 1:1 ratio of AZT to PFA; thus, half the quantity of PFA is delivered when compared to administration of pure PFA, which reduces the chances of the nephrotoxicity from higher doses.

Taken together, our results suggest that N3 is a potential antiviral agent for treatment of CMV infection. Due to lack of evidence of systemic effect in our studies, investigations into bioavailability of N3 when administered by alternate routes will be required. Future research will attempt to optimize dose ranges and delivery routes of N3 to optimize efficacy of CMV inhibition. Methods will be developed to measure serum concentrations and more effective routes of delivery will be explored, including subcutaneous, intravenous, and intramuscular injections. These experiments will attempt to reduce possible toxic effects of N3 and ideally result in more widespread antiviral action.

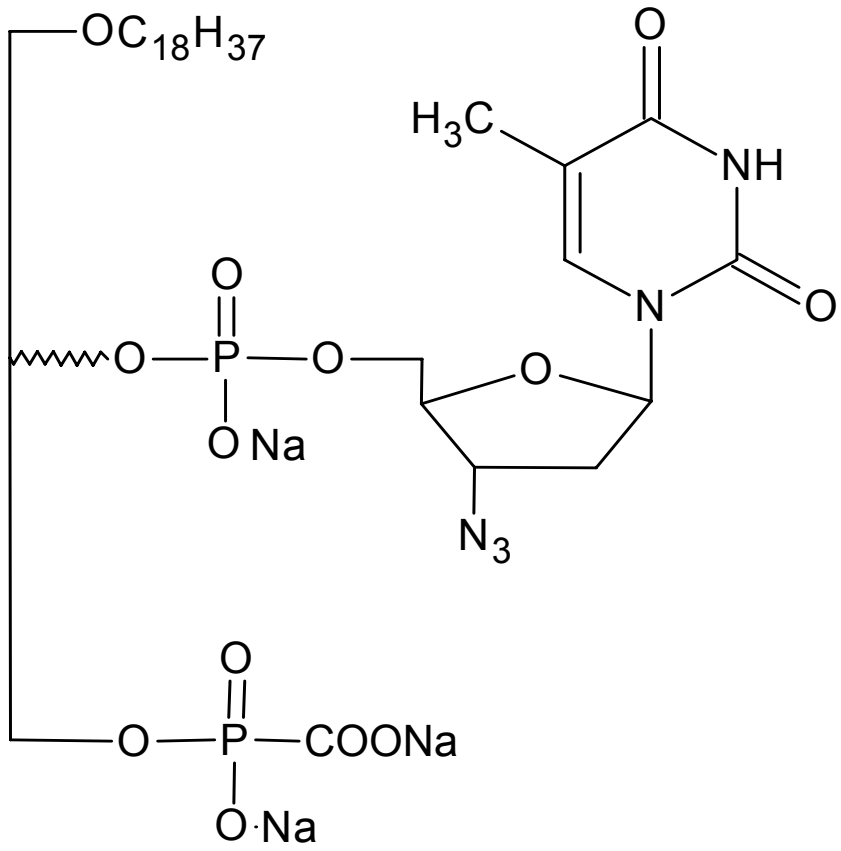

Figure 1. N3: 3'-Azido-2',3'-didesoxy-thymidylyl(5'-2)-1-O-octadecyl-rac-glycerol-3-(hydroxyxcxarbonyl)-phosphonate x3 Na MW=781.34 A duplex antiviral candidate formed by Foscarnet and Zidovudine with a glyceryllipid backbone. N3 is intended for treatment of Cytomegalovirus.

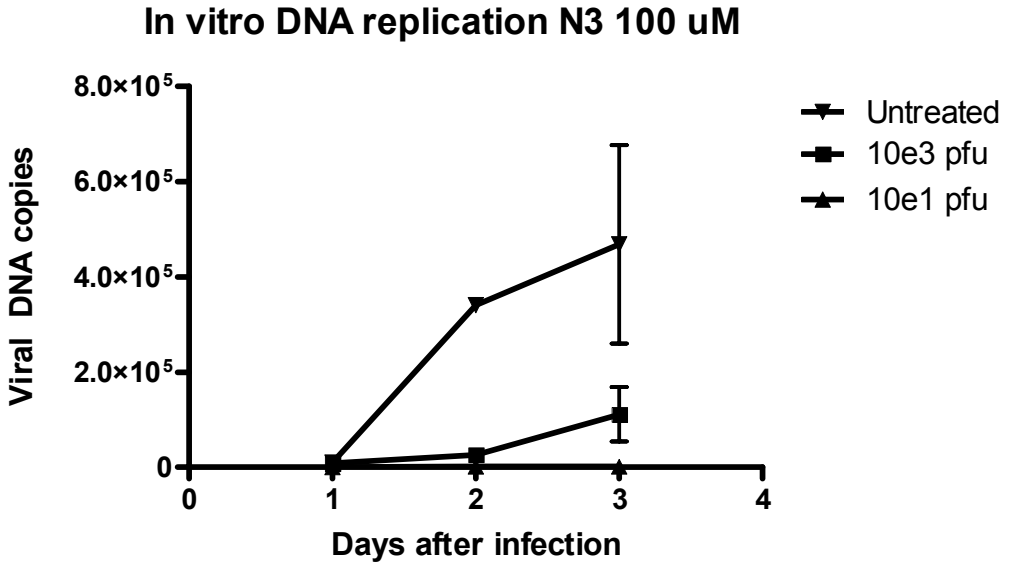

Figure 2. *In-vitro* DNA replication inhibition by N3. Confluent 3T3 fibroblasts were infected with MCMV at concentrations of either 5 X 10^{3} PFU or 5 X 10^{-1} PFU and treated with 100μM of N3. Fibroblasts were harvested at 24, 48, and 72hrs. Samples were homogenized, viral DNA were purified, and quantitated with QRT-PCR. N3 showed inhibition of viral DNA when compared to untreated cells.

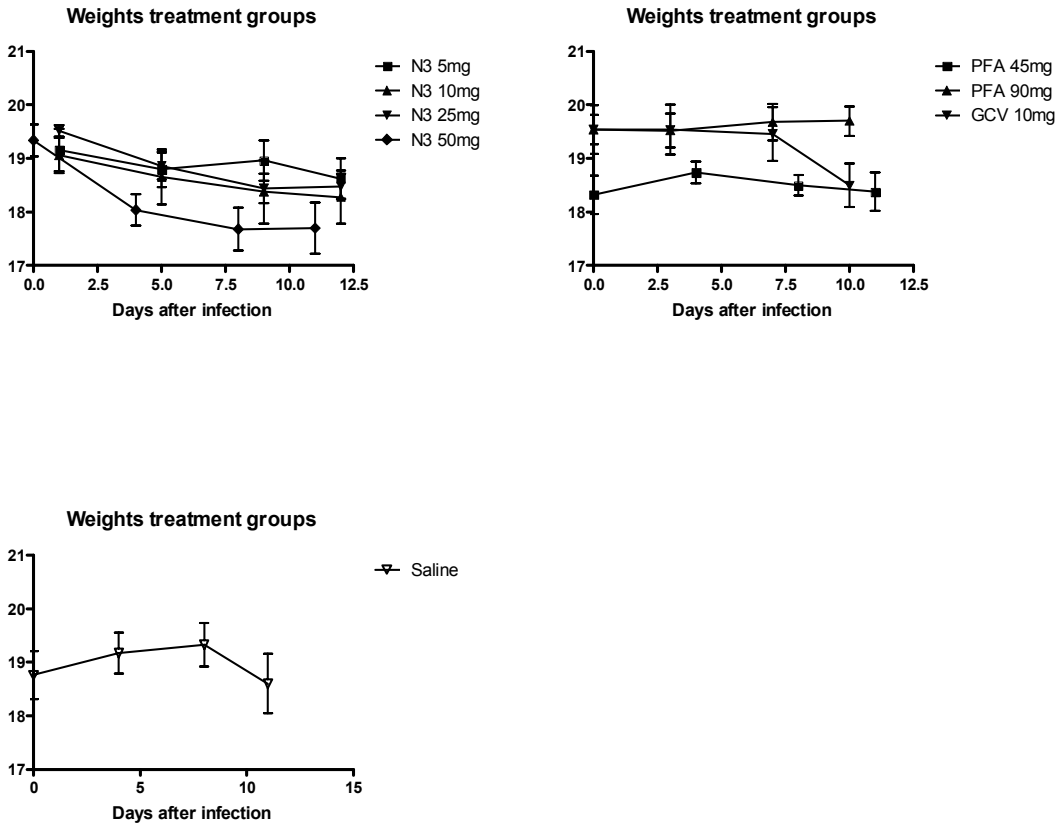

Figure 3. Weight change by group. BALB/c mice were infected with CMV, treated with saline or antivirals. Groups were weighed regularly to evaluate effects of N3 on general metabolism. N3 at 50mg/kg/day caused an approximate 8% total body weight loss. Other groups (PFA, GCV, Saline) showed no significant changes in weight.

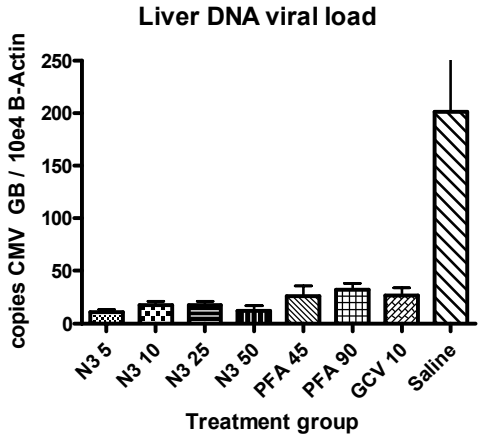

Figure 4. Viral DNA load in the Liver. BALB/c mice were infected with CMV, treated with saline or antivirals, and their livers were harvested 14 days post-infection. Samples were homogenized, DNA were purified, and quantitated with QRT-PCR. ANOVA suggested that treatment groups had significantly lower DNA titers than saline controls ($p=0.0001$), and individual students t-tests comparing each treatment group to saline controls showed that titers were significantly lower for all treatment groups ($p<0.05$). Notably, a dose of 5mg/kg/day of the experimental drug, N3, was as effective in reducing viral load as GCV, PFA, and other higher concentrations of N3.

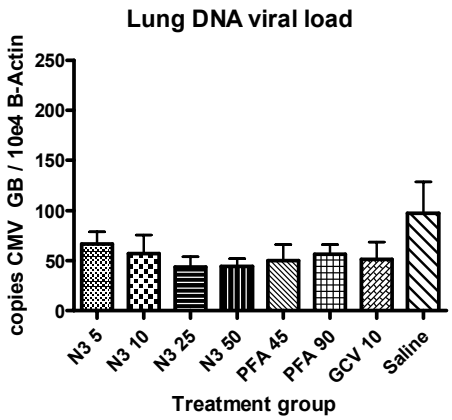

Figure 5. Viral DNA load in the Lung. BALB/c mice were infected with CMV, treated with saline or antivirals, and their lungs were harvested 14 days post-infection. Samples were homogenized, DNA were purified, and quantitated with QRT-PCR. N3 also appeared to have effects comparable to GCV in the lungs ($p=0.48$), as viral DNA quantity was reduced by half in comparison to untreated lungs. There was no significant difference between any treatment group and saline controls in lung viral DNA loads ($p>0.05$).

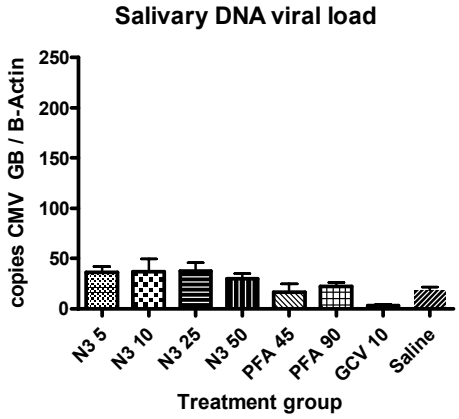

Figure 6. Viral DNA load in the salivary gland. BALB/c mice were infected with CMV, treated with saline or antivirals, and their salivary glands were harvested 14 days post-infection. Samples were homogenized, DNA were purified, and quantitated with QRT-PCR. Individual students t-tests comparing each treatment group to saline controls showed that titers were significantly lower only for the GCV treatment group ($p<0.05$). The experimental drug, N3, appeared ineffective in reducing viral DNA load.

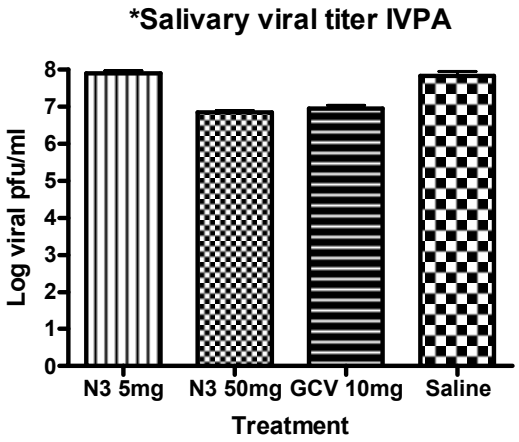

Figure 7. IVPA of the salivary gland. BALB/c mice were infected with MCMV, treated saline or antivirals, and salivary glands were harvested 14 days post infection. Samples were homogenized and applied to confluent 3T3 fibroblasts in culture. Plaque assays of N3 at 5mg/kg/day and 50mg/kg/day were compared to GCV and saline. Plaque assays showed N3 50 mg to have comparable efficacy in reducing infectious particle formation when compared to GCV; and both showed a ten-fold reduction in PFU compared with saline or N3 5mg ($p<0.05$). A low concentration of N3 (5mg/kg) was not effective in reducing infectious particle count.

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